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Artemisia annua L. hot-water extracts show potent activity *in vitro* against Covid-19 variants including delta

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ABSTRACT:

Ethnopharmacological relevance: For millennia, *Artemisia annua* L. was used in Southeast Asia to treat "fever". This medicinal plant is effective against multiple pathogens and is used by many global communities as a source of artemisinin derivatives that are first-line drugs to treat malaria caused by *Plasmodium* parasites.
Aim of the study: The SARS-CoV-2 (Covid-19) global pandemic has killed millions and evolved numerous variants, with delta being the most transmissible to date and causing break-through infections of vaccinated individuals. We further queried the efficacy of *A. annua* cultivars against new variants.
Materials and methods: Using Vero E6 cells, we measured anti-SARS-CoV-2 activity of dried-leaf hot-water *A. annua* L. extracts of four cultivars, A3, BUR, MED, and SAM, to determine their efficacy against five infectious variants of the virus: alpha (B.1.1.7), beta (B.1.351), gamma (P.1), delta (B.1.617.2), and kappa (B.1.617.1).
Results: In addition to being effective against the original wild type (WT) WA1, *A. annua* cultivars A3, BUR, MED, and SAM were also potent against all five variants. IC_{50} and IC_{90} values based on measured artemisinin content ranged from 0.3 to 8.4 μM and 1.4–25.0 μM, respectively. The IC_{50} and IC_{90} values based on dried leaf weight (DW) used to make the tea infusions ranged from 11.0 to 67.7 μg DW and 59.5–160.6 μg DW, respectively. Cell toxicity was insignificant at a leaf dry weight of ≤50 μg in the extract of any cultivar.
Conclusions: Results suggest that oral consumption of *A. annua* hot-water extracts (tea infusions) could potentially provide a cost-effective therapy to help stave off the rapid global spread of these variants, buying time for broader implementation of vaccines.

1. Introduction

The global SARS-CoV-2 (Covid-19) pandemic has infected at least 220 million people and killed greater than 4.5 million (https://coronavirus.jhu.edu/map.html). Numerous variants have rapidly evolved (https://www.who.int/en/activities/tracking-SARS-CoV-2-variants/). The delta variant is currently the most transmissible to date (R_O = 5–7) (Nunes-Vaz and Macintyre, 2021), is a driver of increased rates of hospitalization and moderate to severe disease in unvaccinated individuals, and can cause break-through infections in vaccinated individuals (Gupta et al., 2021). Approved small molecule-based therapeutics are still lacking. Previously, we showed that hot-water extracts of dried leaves of seven cultivars of the medicinal plant, *Artemisia annua* L., used for millennia to treat malaria fever (Hsu, 2006) and sourced from four continents, prevented SARS-CoV-2 replication *in vitro* (Nair et al., 2021). Recently, anti-SARS-CoV-2 efficacy of *A. annua* extracts was independently confirmed (Zhou et al., 2021).

We earlier reported that antiviral efficacy was inversely correlated to artemisinin (ART) content (Nair et al., 2021). Others also observed that compared to *A. annua* L., *A. afra* Jacq. ex Willd., a related perennial species lacking ART, was equally effective against SARS-CoV-2 with IC_{50} values of 0.9–3.4 and 0.65 mg/mL extract, respectively (Nie et al., 2021). Although those results indicated that both *A. annua* and *A. afra* have potent anti-SARS-CoV-2 activity *in vitro* and that the effect was not ART-dependent, it was unclear whether *A. annua* is effective against emerging variants.

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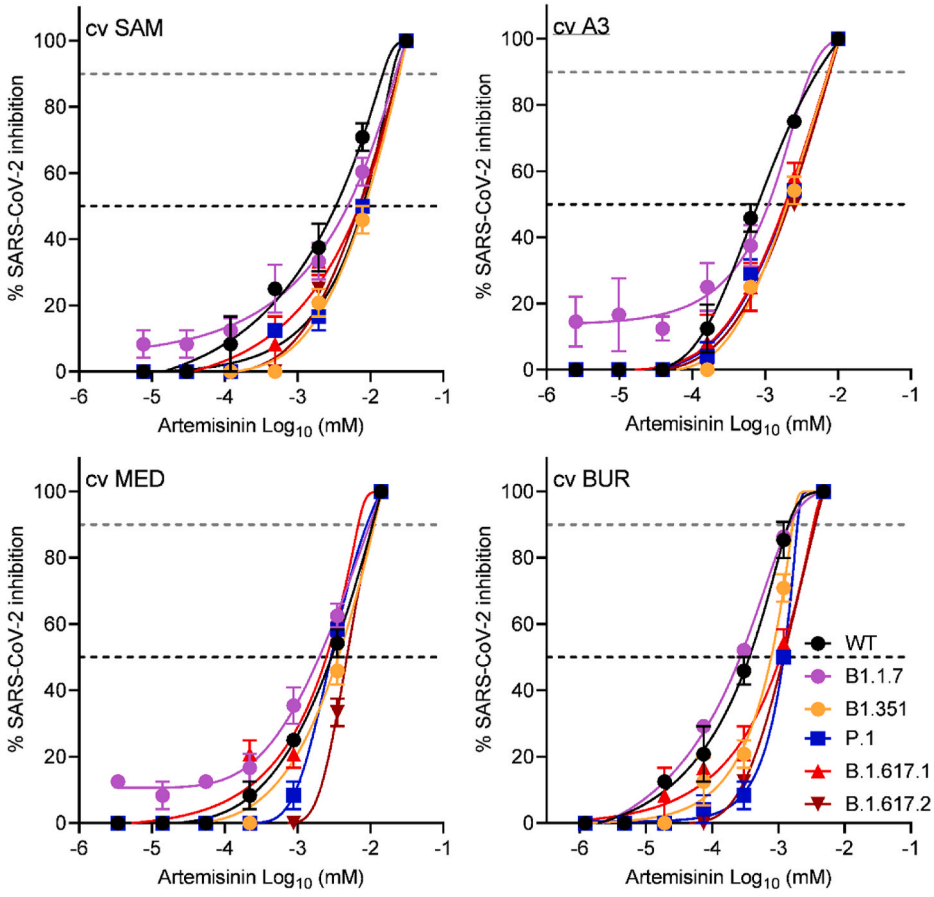

Fig. 1. SARS-CoV-2 variant inhibition by four cultivars of *A. annua* L. hot water extracts normalized to their artemisinin content and compared to WT. WT, USA/WA1; variants: B.1.1.7, alpha; B.1.351, beta; P.1, gamma; B.1.617.1, kappa; B.1.617.2 delta) at a multiplicity of infection (MOI) of 0.1 in Vero E6 cells. Data for alpha and beta variants are extrapolated from Nair et al. (2021). Data are plotted from an average of three replicates ± SE.

Here we report *in vitro* efficacy against three new variants for four of the seven originally studied *A. annua* L. cultivars.

1.1. Methods and materials

2.1 Plant material, extract preparations, and artemisinin analyses: This study used the stored, frozen hot-water extracts (tea infusions) that previously were prepared from dried leaves of *Artemisia annua* L. (SAM, MASS 00317314; BUR, LG0019527; A3, Anamed; MED, KL/015/6407). Details are in Nair et al. (2021). Briefly, hot-water extracts were made from 10 g dried leaves/L that had been boiled in water for 10 min, sieved to remove solids, and filter-sterilized (0.22 μm) prior to storage at −20 °C. For ART analysis, tea infusions were extracted and analyzed by gas chromatography-mass spectrometry as detailed in (Martini et al., 2020). ART contents in μg/mL were: 42.5 for A3; 20.1 for BUR; 59.4 for MED; and 149.4 for SAM (Nair et al., 2021).

2.2 Viral culture and infection: Vero E6 (ATCC CRL-1586) cell cultivation and viral infection were performed as detailed in (Nair et al., 2021). SARS-CoV-2 isolates (USA WA1; alpha, B1.1.7; beta, B1.351; gamma, P.1; delta, B.1.617.2; kappa, B.1.617.1) were sourced from BEI Resources (www.beiresources.org). Viruses were titrated upon propagation to determine their tissue culture infectious dose (TCID) in Vero E6 cells, aliquoted, and frozen at −80 °C until further use. Multiplicity of infection (MOI) was 0.1 as used in other studies (Liu et al., 2020).

2.3 Assays for determining drug inhibition of SARS-CoV-2 and cell viability: Extract dilutions were incubated for 1 h in wells of 96-well tissue culture plates containing a monolayer of Vero E6 cells seeded the prior day at 20,000 cells/well. One hour later SARS-CoV-2 virus was added to each well at a final MOI of 0.1. Cells were cultured for 3 days at 37 °C in 5% CO_2 and were scored for cytopathic effects as previously detailed (Liu et al., 2020). Results were converted into percent of control. Drug concentrations were log transformed. Concentration of drug (s) that inhibited virus by 50% (*i.e.*, IC_{50}), and concentration of drug(s) that killed 50% of cells (*i.e.*, CC_{50}), were determined via nonlinear logistic regressions of log(inhibitor) versus response-variable dose-response functions (four parameters) constrained to a zero-bottom asymptote by statistical analysis. Viability of Vero E6 cells post extract treatment was already reported in Nair et al. (2021) for the same extracts. Dry weight of leaves and total ART content measured in the infusion extracts were reported in Nair et al. (2021) and were used to normalize the IC_{50} and IC_{90} values for the new variants tested or the WT and variants tested previously.

2.4 Chemicals and reagents: Reagents were from Sigma-Aldrich (St. Louis, MO). EMEM (Cat # 30–2003) and XTT reagent (Cat # 30-1011 k) were from ATCC. Renilla-Glo was from Promega (E2720).

2.5 Statistical analyses: All *in vitro* anti-SARS-CoV-2 analyses were done at least in triplicate. Plant extract analyses had n ≥ 6 independent assays. IC_{50} and IC_{90} values were calculated using GraphPad Prism V9.2.

1.2. Theory/calculation

A. annua hot-water infusions already shown to be effective against SARS-CoV-2 are also effective against newly emerging variants.

2. Results and discussion

A. annua hot-water extracts inhibited the recently evolved variants of SARS-Cov-2 (Fig. 1) with calculated IC_{50} values normalized to the ART

Table 1
Potency of *A. annua* L. hot-water extracts (10 g/L) against 6 strains of SARS-CoV-2 based on either artemisinin content or leaf dry weight (DW).

Cultivar	Potency normalized to artemisinin content (μM)											
	IC_{50} μM artemisinin						IC_{90} μM artemisinin					
	WA1[a]	B.1.1.7[a]	B.1.351[a]	P.1	B.1.617.1	B.1.617.2	WA1[a]	B.1.1.7[a]	B.1.351[a]	P.1	B.1.617.1	B.1.617.2
SAM	3.4	4.9	8.4	7.9	7.0	7.0	14.9	22.3	25.0	20.0	24.8	24.1
A3	0.8	1.1	2.0	1.9	1.9	2.1	5.2	4.2	7.4	6.5	7.3	7.8
BUR	0.4	0.3	0.8	1.2	1.1	1.2	1.4	1.5	1.6	1.9	3.4	3.6
MED	2.9	2.0	3.6	2.9	2.5	4.8	10.7	9.8	11.3	8.9	6.8	10.6
Potency normalized to dry mass of leaves used in tea infusion (μg)												
	IC_{50} μg leaf DW						IC_{90} μg leaf DW					
	WA1	B.1.1.7	B.1.351	P.1	B.1.617.1	B.1.617.2	WA1	B.1.1.7	B.1.351	P.1	B.1.617.1	B.1.617.2
SAM	21.5	31.3	53.7	50.7	45.0	45.1	95.6	143.1	160.4	128.0	159.3	154.7
A3	15.7	22.1	39.6	38.2	37.0	42.4	103.7	84.1	147.9	150.2	145.3	154.7
BUR	15.1	11.0	32.5	50.1	44.7	49.8	59.5	61.2	68.6	80.4	143.3	150.4
MED	41.7	28.2	51.5	41.0	37.0	67.7	152.5	139.7	160.6	127.0	145.3	151.6

IC_{50} and IC_{90} are values where virus is 50% and 90% inhibited. Data are an average of three replicates.
[a] Values taken from Nair et al. (2021).

content of each tea infusion ranging from 1.1 to 7. 9 μM for the gamma, delta, and kappa variants. Although already reported by (Nair et al., 2021), WT(WA1), alpha, and beta variants were included for direct experimental comparison (Fig. 1; Table 1). The lowest IC_{50} values were from the BUR cultivar and the highest were from the SAM cultivar. As previously shown (Nair et al., 2021), there was an inverse correlation between ART in extracts and antiviral efficacy. The lowest ART content (BUR) yielded the greatest potency (the lower the IC_{50}, the more potent the drug/extract), providing evidence that ART is not the only active antiviral agent in these extracts. Nie et al. (2021) further validated that ART was not the only anti-SARS-CoV-2 compound in the extracts by showing that aqueous extracts of the PAR cultivar of *Artemisia afra*, an *Artemisia* species lacking ART, had an IC_{50} of 4.1 mg/mL, within the range of 3.1–13.0 mg dried extract/mL of the *A. annua* cultivars studied therein. As already reported for extracts used in this study, no cytotoxicity was observed at a dry weight of ≤50 μg in the extract of any cultivar (Nair et al., 2021).

Although Zhou et al. (2021) also showed that *A. annua* hot-water extracts had anti-SARS-CoV-2 efficacy, it was difficult to compare their IC_{50} values because they did not test the same viral strain, use the same plant cultivars, or make their extracts and apply them to virus-infected cells using the same procedures. Using *A. annua* aqueous extracts against the BavPat January 2020 strain of SARS-CoV-2 in Vero E6 cells, the IC_{50} values were 390 and 260 μg dried extract/mL for pretreated and treated cells, respectively. For pretreatment, extract was added 1.5 h before virus infection and for treatment, drug was added 1 h after virus infection. Ethanolic extracts yielded IC_{50} values about 50% lower, and thus were more potent than the aqueous extracts. To compare results of both studies, we calculated the dry mass of leaves equivalent to their reported IC_{50} to be 941.2 mg. The IC_{50} mass reported in (Nair et al., 2021) ranged from 13.5 to 57.4 μg, varying by cultivar. The leaf dry mass IC_{50}s in this study for the gamma, delta, and kappa variants ranged from 38.2 to 50.7, 42.4–67.7, and 37.0–45.0 μg leaf DW, respectively. The three orders of magnitude difference between this study and Zhou et al. (2021) likely result from the above noted differences in methodology.

ART and its derivatives have some anti-SARS-CoV-2 activity (Cao et al., 2020; Gendrot et al., 2020a, 2020b; Nair et al., 2021; Zhou et al., 2021). However, in those reports where there are direct comparisons with *Artemisia* extracts, ART is not the only active phytochemical, suggesting there are other antiviral compounds in the plant. *A. annua* contains a rich assortment of identified phytochemicals (Ferreira et al., 2010), some of which have activity against human coronavirus proteins. For example, quercetin and myricetin have inhibitory activities against SARS-CoV NTPase/helicase with IC_{50}s of 0.1 and 2.7 μM, respectively, and luteolin has an IC_{50} of 10.6 μM against SARS-CoV in Vero E6 cells (Russo et al., 2020). Investigating other potential anti-SARS-CoV-2 phytochemicals found in *A. annua* and *A. afra* is warranted.

3. Conclusions

Hot-water (tea infusion) extracts of *A. annua* are active against SARS-CoV-2 and its variants alpha, beta, gamma, delta, and kappa. In our original report, anti-SARS-CoV-2 activity inversely correlated with ART content. Herein, similar responses are noted for gamma, delta, and kappa variants wherein the *A. annua* cultivar with the lowest ART content, BUR, generally had the lowest (most effective) IC_{50}. These results demonstrate the potential of the extracts as treatments in the global fight against this constantly evolving virus. We urge WHO to consider including extracts and encapsulated dried leaves in their announced clinical trials that already include artesunate (Kupferschmidt, 2021). We aim to test preclinical models of SARS-CoV-2 in rodent models (Dinnon et al., 2020; Gu et al., 2020) that could help advance *A. annua* as an inexpensive therapeutic in parts of the world where logistic issues such as delivery require longer time to achieve vaccination levels that would ultimately quell this pandemic.

Declaration of competing interest

Authors declare they have no competing conflicts of interest in the study.

Author contributions

Manoj Nair: Conceptualization; Data curation; Formal analysis; Investigation; Writing - review & editing.
Yaoxing Huang: Data curation; Formal analysis; Investigation.
David Fidock: Data curation; Resources; Writing - review & editing.
Melissa Towler: Resources; Writing - review & editing.
Pamela Weathers: Conceptualization; Resources; Roles/Writing - original draft; Writing - review & editing.

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